Western Blot for the diagnosis of the acute and chronic phase of animal and human fasciolosis, using different antigens of *Fasciola hepatica*

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Abstract

Fasciolosis is a zoonotic infection of high prevalence in Peru, being Fasciola hepatica the parasite responsible for this disease recognized as a public health emergency; this parasite infects humans and animals; human transmission occurs in rural Andean populations dedicated to agriculture becoming even hyperendemic in the poorest areas of Peru where immunoenzymatic and immunoelectrotransfer diagnostic techniques, currently considered as the most sensitive and specific in detecting the parasite, are not applied; however, research in this regard concludes that through the application of these techniques, some specific proteins have been determined from the second week of the chronic phase of the disease, thus making it essential to apply diagnostic methods that identify specific proteins of F. hepatica in the acute phase of the disease, even when the antigen is found in low concentration in the sample, so that, unlike routine diagnosis, the Western Blot is an effective alternative for the diagnosis of animal and human fasciolosis. Therefore, the aim of this review was to know the application of the Western Blot technique for the diagnosis of fasciolosis in the acute and chronic phase. The advantages and disadvantages of this technique have been

discussed, as well as its potential in specific antigen recognition, all compiled from state-of-the-art scientific literature, considering the timely detection essential for the respective treatment in animals and humans.

Keywords: *Fasciola hepática, Fasciolosis, Western blot, Antigens.*

INTRODUCTION

Parasitosis has been a global Public Health problem since immemorial (Botero & Restrepo, 2005; M. del P. Díaz et al., 2013; Morales, 2020). It is transmissible under natural conditions between vertebrate and invertebrate animals and man (Steinfeld et al., 2009; Szyfres, 2003), one of these parasitoses being fasciolosis, which is found distributed in Europe, Asia, the Middle East, and Latin America (L. Marcos et al., 2007).

The disease is caused by F. hepatica, which affects cattle, sheep, and goats (Uribe et al., 2012; Valderrama, 2016). It is also known that in all world countries, liver flukes of the genus Fasciola cause fasciolosis (Valero et al., 2018). The importance of this disease focuses on several aspects, among them the significant economic losses in South American countries with a prevalence of 25% in dairy cattle (Correa et al., 2016).

In Latin America, new endemic areas of human beings affected by fasciolosis have been described (Valero et al., 2018). The disease caused by this parasite represents a global prevalence of 24.3% in Peru. In the Peruvian highlands in 2002, 71.4% were reported with positive serology for 2004, with the Department of Puno considered one of the regions with higher prevalence (Corral-Ruiz & Sánchez-Torres, 2020). This disease presents two critical phases; the acute phase, where there is evidence of mechanical irritation suffered by the peritoneum, liver tissue, and allergic and toxic reactions (Uribe et al., 2012), while in the chronic phase, there is diarrhea, sometimes steatorrhea, fever, pain in the right hypochondrium and weight loss. Therefore, it is essential to identify specific diagnostic antigens in each disease phase (Cruz y López et al., 2016).

Worldwide, there are standardized techniques for the diagnosis of fasciolosis, such as ELISA, Dennis (Correa et al., 2016), stool tests, and necropsy, among others (Sanchís et al., 2015); however, they are not very precise, which would lead to the use of techniques capable of identifying specific antigens of F. hepatica for an opportune diagnosis (K. Davelois et al., 2016).

Detecting fasciola antigens in humans and animals is considered a good alternative for the immunodiagnosis of infection by this parasite, making possible the creation of a vaccine (Meshgi et al., 2008). Serological diagnosis is essential, based on antigenic fractions of the parasite, since it can be used for the early diagnosis of human fasciolosis (Shafiei et al., 2015). On the other hand, the Western Blot (WB) technique is being increasingly used in the diagnosis of parasitic diseases due to its high specificity, which lies in the recognition of antigens (Arteaga-García & Jara, 2013; Jara Campos et al., 2018; Parisaca & Calla, 2018). This is considered opportune for the diagnosis of this parasitosis in order to guarantee public health (Rodríguez et al., 2019).

In this sense, the main objective of this research was to carry out a critical study about the advantages and disadvantages of the Western Blot Technique for the diagnosis of the acute and chronic phases of animal and human fasciolosis using different F. hepatica antigens, contributing to public health.

Method

Search procedure and strategies

The article is a systematic review based on the PRISMA methodology (Moher et al., 2015). First, a search for scientific research was carried out in different electronic resources. Specifically, the databases used were Scopus, Web of Science, and Scielo. Then, the following search formula was used in English and Spanish: (Fasciolosis) AND (Fasciola hepatica) AND (Western blot) AND (Antigens). Subsequently, a second search was carried out in the Scielo database. As a complement, a search for information in English and Spanish was also made in Google Scholar, always recognizing reliable sources. All these searches were carried out during the months of January to March 2022.

The information obtained corresponds to primary, secondary, and tertiary sources. In total, 642 scientific documents were obtained, of which the PRISMA selection criteria discarded 591.

Inclusion and exclusion criteria

The inclusion criteria considered: works in English and Spanish, empirical studies; laboratory work associated with the Western blot technique. The established exclusion criteria were: papers published in a language other than English or Spanish, techniques used other than Western blot; qualitative research.

Body and discussion

3.1 Hepatic Fasciola

Fasciolosis is a parasitic infection caused by flukes of the genus Fasciola, and the F hepatica specie is the most frequent worldwide. This specie is distributed on all continents and infects many mammals, mainly sheep, cattle, goats, and men (Mas-Coma, 2005). This leads to a considerable affection for livestock, generating significant economic losses (Alzamora-Gonzales et al., 2016).

3.1.1 Phases of fasciolosis

Hepatic fasciolosis can present mainly acutely and chronically. The clinical manifestations are generally non-specific and vary according to the phase of the disease (L. A. Marcos et al., 2009).

Acute phase

At this stage, most studies agree on the difficulty of diagnosing fasciolosis. Thus, in Peru, where this disease is endemic, from 1963 to 2005, a total of 1701 cases were reported, of which only 11% were diagnosed in the invasion phase and 89% in the state phase (Cruz y López et al., 2016; Espinoza et al., 2010).

Other investigations, theoretically, affirm that the acute phase of the infection by F. hepatica requires serological methods for its detection. The Western Blot is an excellent option because other techniques do not detect the disease due to the absence of eggs that are removed by the parasite intermittently (Abdolahi Khabisi et al., 2017; Espinoza et al., 2010; Jiménez et al., 2001; Náquira-Velarde, 1995; Wongkham et al., 1999).

Chronic phase

Investigations carried out in the chronic phase of the disease indicate that Western Blot quickly detects them to search for antibodies against F. hepatica (Cruz y López et al., 2016; Khan et al., 2017). In the last century, studies considered the stool test the gold standard for detecting F. hepatica (Muñoz Zambrano et al., 2020); however, it is applicable only during the chronic phase of fasciolosis since, during the acute phase, it could report false negatives (Muñoz Zambrano et al., 2020).

On the other hand, in the pathogenesis of this disease, there are two main periods: the first, called initial or invasion, which begins from the moment of ingestion of the metacercariae until the implantation of the parasites in the bile ducts, and the second period, which is known as the stage and is when the parasites reach sexual maturity and begin to eliminate eggs in the fecal matter of infected humans or animals (Espino, 1997; Pérez, 2018).

3.2. Diagnosis

3.2.1. Sample collection and serum extraction

Human population samples

The selection of the subjects can be coordinated with the personnel of the health establishments and select individuals of different ages that show characteristic symptoms of fasciolosis. Each individual is extracted 5 ml of venous blood, centrifuged, and frozen until processing (Cornejo et al., 2010).

Animal samples

Adult fasciola worms should be collected from the bile ducts of infected cattle at local slaughterhouses (Abdolahi Khabisi et al., 2017) and washed approximately five times with phosphate-buffered saline (PBS 1X; pH: 7.2) to remove exudates from the bile ducts. From each infected liver, transfer three to four worms to 70% alcohol and the same to 1X PBS (pH: 7.2) to prepare the antigen (Abdolahi & Sarkari, 2016).

Extraction of animal and human serum

To obtain animal serum, blood samples are taken naturally before the sacrifice of those infected animals; The samples are taken to the laboratory and centrifuged at 2500 rpm for 5 minutes. With the help of vials, the serum is separated from the blood (Walsh et al., 2021). Positive and negative sera are taken from cattle for analysis (R. Sierra et al., 2017).

Sera from human beings must have informed consent and be processed under conditions of anonymity. Protocols established in the ethics committee of each institution must be used (K. Davelois et al., 2016) as positive controls for human fasciolosis, reactive and negative sera from the urban area of the city (R. Sierra et al., 2017). It is important to consider patients in the chronic phase (who have found eggs in the feces) whose samples have been processed by rapid sedimentation (K. Davelois et al., 2016).

3.2.1.1. Antigen production

Adult flukes are collected from infested livers and washed 3-5 times at room temperature with saline solution for one hour. Then, the prepared antigens are centrifuged for 20 min, being stored at -70 °C until use (Meshgi et al., 2008).

By immunohistochemistry, it is known that the FhTP16.5 antigen of the adult fluke of F. hepatica localizes specifically on the surface of the integument at various stages of development and in parenchymal tissues, which is very attractive for chemotherapy and immunoprophylaxis, in addition to the fact that this antigen is capable of inducing antibodies detected in the serum of humans with chronic fasciolosis and rabbits at 3 to 12 weeks after infection (Gaudier et al., 2012).

Somatic antigen

For this procedure, adult Fasciola worms should be used, which are homogenized in 1X PBS using a tissue grinder. At the same time, an anti-protease cocktail is required during the procedure. After homogenization, the sample was centrifuged at 13,000 g for 30 min at 4°C. The supernatant should be removed and stored at -20°C until use (Abdolahi & Sarkari, 2016).

To obtain antigens, samples must be treated with 0.01M Tris-HCl pH 8.0; 1% sodium dodecyl sulfate, 6% glycerol and 1% bromophenol blue, and 2.5M dithiothreitol, then they must be heated at 65ºC for 20 min (Abdolahi & Sarkari, 2016). Studying the behavior of antigens is a crucial objective for the future diagnosis, treatment, and development of vaccines for fasciolosis (Xu et al., 2020).

To perform this somatic lysate procedure, it is necessary to obtain pure flukes, wash 3 times with PBS 1X pH 7.2, then sonicate, then perform a thermal shock, centrifuge at 3000 rpm at 4ºC for 10 minutes twice, store the supernatant at -70 ºC until its use (Cornejo et al., 2010).

Excretory-secretory antigen

This antigen (AgE/hSF) is vital in stimulating the antibody response before the onset of symptoms. It has been used in different immunodiagnostic techniques with good sensitivity (S) and specificity (E) results (R. Sierra et al., 2017). They are obtained through in vitro culture in Eagle's minimum essential medium, for which viable adult forms are required (K. Davelois et al., 2016). Several specimens of F. hepatica are placed in a sterile Petri dish with 100ml of 1X PBS (pH 7.2), incubated at 37°C for approximately four hours, and refrigerated at 4°C overnight. Parasites are removed, and 1X PBS (excretion-secretion antigen) is collected. The supernatant is distributed in cryovials and frozen at -80°C (Cornejo et al., 2010).

It should be considered that the sensitivity and specificity are higher when working with this antigen than when working with somatic antigen (Cornejo et al., 2010). In addition to evaluating secretion-excretion antigens in bovine feces samples using the immunological methods of ELISA, agglutination in latex (AL) and indirect hemagglutination (HAI) and its correlation with the Dennis coproparasitological method (CD), it was determined that ELISA-Fascidig would enable the diagnosis of fasciolosis during the prepatent and patent phase of the disease with a significant correlation with the coproantigen (Colona et al., 2014).

Membrane antigen

Bile duct parasites should be collected from infected cattle from a local slaughterhouse no later than 2 hours post-slaughter to prepare membrane antigens. The parasites were washed in prewarmed 1X PBS, then placed in 1 mL of sterile medium with antibiotic (Walsh et al., 2021).

3.3 Western blotting (WB)

It is a semiquantitative technique where cell and molecular biology principles are applied since it allows the identification of the specific protein profile from a complex mixture of proteins extracted from cells (Mahmood & Yang, 2012; R. Sierra et al., 2017).

From its origin in the seventies to the present, this technique has reached a fundamental level in diagnosing various medical conditions and is a daily tool in biomedical research (Escalante et al., 2011; Martínez et al., 2017; M. F. Sierra et al., 2020). Thus, in the clinical field, it is a diagnostic method for various autoimmune, oncological, and infectious diseases, including fasciolosis (Martínez et al., 2017). Additionally, it presents advantages over other serological techniques on the market that are not affordable in endemic areas due to the high cost, the requirement of additional equipment, and the process and time of completion (Escalante et al., 2011).

The technique is based on separating proteins in polyacrylamide gels (SDS-PAGE) according to their charge and molecular weight for their subsequent transfer and absorption on a solid nitrocellulose membrane, in which a specific antibody detects the protein allowing the recognition of antibodies by antigens (K. Davelois et al., 2016; K. R. Davelois et al., 2019; Jara Campos et al., 2018). Antigen-antibody binding is detected by adding an antibody that recognizes the constant fraction of human immunoglobulin, which is coupled to an enzyme (Escalante et al., 2011). This technique has already been applied in humans (children, adolescents, and adults). Also, it has been applied in animals (cattle, sheep, pigs, birds, horses, and rodents) (Arteaga-García & Jara, 2013; Gönenç et al., 2004), showing high specificity and sensitivity in relation to other techniques such as ELISA, coproparasitology, Arc 2, among others.

Procedure

To detail the sequence of steps, what is described by Xu et al. (2020) will be followed:

The obtained antigens were treated with 0.01M Tris-HCl pH 8.0; 1% sodium dodecyl sulfate; 6% glycerol and 1% bromophenol blue, and 2.5M dithiothreitol, to then be heated at 65 ºC for 20 minutes, thus achieving a more stable binding between SDS and protein.

The final concentration of the antigen is 0.2 µg/uL. In vertical electrophoresis, the different antigens were placed in the wells in an amount equivalent to 1 µL per mm of gel width. The runs are performed on 8.3 x 7.0 x 0.075 cm polyacrylamide minigels at the concentration of 15% in the resolution gel and 3% in the concentrator gel using molecular weight marker (low weight range) in the Mini Proten tetra cell and run at 200V for 60 min from 2 to 8ºC.

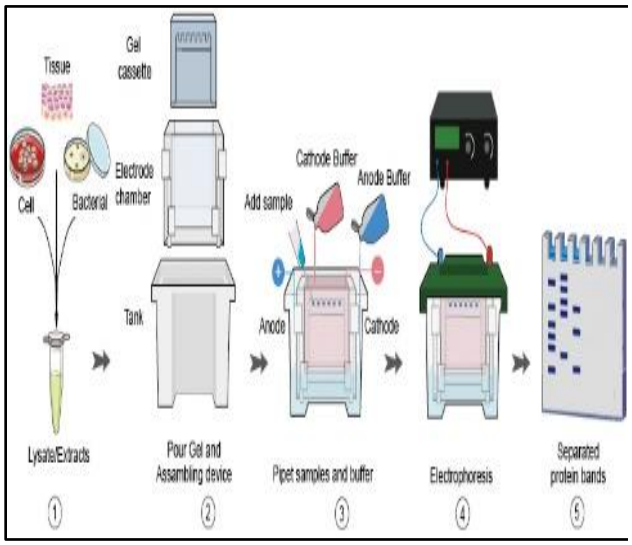
Electrotransfer

Transfer of the proteins from the gel to a 0.2 µm nitrocellulose membrane (Figure 1) is performed using transfer buffer (0.2M Tris-HCl, 20% methanol, and ultrapure water) in the Trans Blot Cell. at 100V, 2A for 90 min. The best antigen is selected to be run on the entire surface of the minigel and then cut into 3 mm wide strips to be evaluated in eight-channel incubation plates.

Blocking and activation of antibodies

For each of the sera, a 1/50 dilution is made in PBS 1X-tween 20 and skimmed milk powder at a 1/20 dilution as a blocker of the areas of the paper not occupied by antigens; They are incubated under constant agitation at room temperature for one hour. After being washed with PBS 1X-tween 20, they are placed in an enzyme conjugate solution (anti-human IgG labeled with peroxidase) at a dilution of 1/1000 for one hour. After five washes, the antigens are revealed with a substrate (0.01% H2O2 and 0.5 mg/ml diaminobenzidine) for 10 min, stopping the reaction with ultrapure water for 5 min. Finally, the membrane is incubated for 1 to 2 minutes and visualizes the result in a photodocumenter, preferably in dark conditions.

Figure 1. Western Blot technique for the diagnosis of fasciolosis



Source: (Mahmood & Yang, 2012).

Advantages and scientific reports

The advantages that this technique represents have been seen in studies carried out in birds indicating the detection of specific antibodies, IgG/WB, with sensitivity and specificity greater than 90% (Arteaga-García & Jara, 2013; Escalante et al., 1995; Yoosefy et al., 2018). The Western Blot technique concerning other techniques, has demonstrated its ability to detect early positivization in the acute phase when eggs are not yet detectable in stools (Arteaga-García & Jara, 2013), and considerably reduces cross-reactions with other trematodes (Gönenç et al., 2004), in sheep they demonstrate that the early phase can be detected (two weeks) after infection by F. hepatica (Gönenç et al., 2004) and is evidenced by the presence of bands of 24, 33 and 66 kDa revealed in the serum of all sheep detected with F. hepatica, using the crude antigen prepared (Gönenç et al., 2004) and in turn, these bands were not detected in any of the 20 sera negative for F. hepatica.

On the other hand, the secretory/excretory antigen prepared from F. hepatica determines three specific bands of 33, 39.5, and 42 kDa also found in the serum of all sheep infected with F. hepatica; however, these bands were not detected in any of the negative sera. In diagnosing rodents, this technique determined the disease in the first week of infection (L. A. Díaz et al., 1998). A seroprevalence of 7.4% was reported compared to 2.3% detected by coproparasitology (K. R. Davelois et al., 2019). In a cross-sectional study, the diagnostic performance and sensitivity of the Western Blot test against secretory excretory antigens of adult flukes of F. hepatica was evaluated to probe with sera from the patient infected with the parasite, obtaining 2 glycoproteins GP 17 and 23 kDa for fasciolosis detecting the presence of antibodies with a sensitivity of 96% (K. Davelois et al., 2016), which confirms the advantage of Western Blotting. Research reveals that the Western Blot technique is more reliable than other techniques, such as ELISA, due to the characteristics of recognizing specific bands of F. hepatica proteins (Acici et al., 2017), as well as specific protein fractions up to 100% (Ramos, 2013; R. Sierra et al., 2017).

Disadvantages

One of the disadvantages of this technique is that, in completed investigations, standardization errors are observed, leading to low performance of the technique's specificity. In addition, the genetic variability of F. hepatica can lead to unclear results. However, this could be solved with trained personnel and genomics studies.

Conclusion

Fasciolosis is a disease that has human beings and vertebrate animals as its definitive host. This represents a necessary problem to treat

since it causes negative consequences in different sectors (health, economy, and livestock). Early detection would help to generate immediate treatments for its control. Therefore, the diagnosis using the Western Blot technique has promising potential. This undoubtedly lies in the advantages generated (high specificity, early detection, and high sensitivity). This detailed study of the scientific literature has revealed the contributing role of this technique with different vertebrates and humans, obtaining high-reliability values. This leads to the fact that it is a profitable and effective technique, which gives the certainty that the Wertern Blot technique, at present, can be considered the gold standard for the detection of fasciolosis in its acute and chronic phases, all referred to as the recognition of the antigens of this parasite.

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